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NOVEL POTASSIUM CHANNEL MOLECULES AND USES THEREFOR

Related Applications

This application claims priority to U.S. Patent Application No.: 09/431,367, filed on November 1, 1999 and U.S. Patent Application No.: 09/259,951, filed on March 1, 1999, incorporated herein in their entirety by this reference.

Background of the Invention

Potassium (K^+) channels are ubiquitous proteins which are involved in the setting of the resting membrane potential as well as in the modulation of the electrical activity of cells. In excitable cells, K^+ channels influence action potential waveforms, firing frequency, and neurotransmitter secretion (Rudy, B. (1988) *Neuroscience*, 25, 729-749; Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd Ed.). In non-excitable cells, they are involved in hormone secretion, cell volume regulation and potentially in cell proliferation and differentiation (Lewis *et al.* (1995) *Annu. Rev. Immunol.*, 13, 623-653). Developments in electrophysiology have allowed the identification and the characterization of an astonishing variety of K^+ channels that differ in their biophysical properties, pharmacology, regulation and tissue distribution (Rudy, B. (1988) *Neuroscience*, 25, 729-749; Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd Ed.). More recently, cloning efforts have shed considerable light on the mechanisms that determine this functional diversity. Furthermore, analyses of structure-function relationships have provided an important set of data concerning the molecular basis of the biophysical properties (selectivity, gating, assembly) and the pharmacological properties of cloned K^+ channels.

Functional diversity of K^+ channels arises mainly from the existence of a great number of genes coding for pore-forming subunits, as well as for other associated regulatory subunits. Two main structural families of pore-forming subunits have been identified. The first one consists of subunits with a conserved hydrophobic core containing six transmembrane domains (TMDs). These K^+ channel α subunits participate in the formation of outward rectifier voltage-gated (Kv) and Ca^{2+}-dependent K^+ channels. The fourth TMD contains repeated positive charges involved in the voltage gating of these channels and hence in their outward rectification (Logothetis *et al.* (1992) *Neuron,* 8, 531-540; Bezanilla *et al.* (1994) *Biophys. J.* 66, 1011-1021).

The second family of pore-forming subunits have only two TMDs. They are essential subunits of inward-rectifying (IRK), G-protein-coupled (GIRK) and ATP-sensitive (K_{ATP}) K^+ channels. The inward rectification results from a voltage-dependent block by cytoplasmic Mg^{2+} and polyamines (Matsuda, H. (1991) *Annu. Rev. Physiol.*, 53, 289-298).

A conserved domain, called the P domain, is present in all members of both families (Pongs, O. (1993) *J. Membr. Biol.*, 136, 1-8; Heginbotham *et al.* (1994) *Biophys. J.* 66,1061-1067; Mackinnon, R. (1995) *Neuron*, 14, 889-892; Pascual *et al.*, (1995) *Neuron.*, and 14, 1055-1063). This domain is an essential element of the aqueous K^+-selective pore. In both groups, the assembly of four subunits is necessary to form a functional K^+ channel (Mackinnon, R. (1991) *Nature*, 350, 232-235; Yang *et al.*, (1995) *Neuron*, 15, 1441-1447.

In both six TMD and two TMD pore-forming subunit families, different subunits coded by different genes can associate to form heterotetramers with new channel properties (Isacoff *et al.*, (1990) *Nature*, 345, 530-534). A selective formation of heteropolymeric channels may allow each cell to develop the best K^+ current repertoire suited to its function. Pore-forming α subunits of Kv channels are classified into different subfamilies according to their sequence similarity (Chandy *et al.* (1993) *Trends Pharmacol. Sci.*, 14, 434). Tetramerization is believed to occur preferentially between members of each subgroup (Covarrubias *et al.* (1991) *Neuron*, 7, 763-773). The domain responsible for this selective association is localized in the N-terminal region and is conserved between members of the same subgroup. This domain is necessary for hetero- but not homomultimeric assembly within a subfamily and prevents co-assembly between subfamilies. Recently, pore-forming subunits with two TMDs were also shown to co-assemble to form heteropolymers (Duprat *et al.* (1995) *Biochem. Biophys. Res. Commun.*, 212, 657-663. This heteropolymerization seems necessary to give functional GIRKs. IRKs are active as homopolymers but also form heteropolymers.

New structural types of K^+ channels were identified recently in both humans and yeast. These channels have two P domains in their functional subunit instead of only one (Ketchum *et al.* (1995) *Nature*, 376, 690-695; Lesage *et al.* (1996) *J. Biol. Chem*, 271, 4183-4187; Lesage *et al.* (1996) *EMBO J.*, 15, 1004-1011; Reid *et al.* (1996) *Receptors Channels* 4, 51-62). The human channel called TWIK-1, has four TMDs. TWIK-1 is expressed widely in human tissues and is particularly abundant in the heart and the brain. TWIK-1 currents are time independent and inwardly rectifying. These properties suggest that TWIK-1 channels are involved in the control of the background K^+ membrane conductance (Lesage *et al.* (1996) *EMBO J.*, 15, 1004-1011).

Table of Contents

Summary of the Invention

The present invention is based, at least in part, on the discovery of novel members of the TWIK (for **T**andem of P domains in a **W**eak **I**nward rectifying K^+ channel) family of potassium channels, referred to herein as TWIK-2, TWIK-3, TWIK-4, and TWIK-5 nucleic acid and protein molecules. The TWIK-2, TWIK-3, TWIK-4, and TWIK-5 molecules of the present invention are useful as targets for developing modulating agents to regulate a variety of cellular processes. Accordingly, in one aspect, this invention provides isolated nucleic acid molecules encoding TWIK proteins or biologically active portions thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of TWIK-encoding nucleic acids.

In one embodiment, a TWIK nucleic acid molecule of the invention is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% or more identical to the nucleotide sequence (*e.g.*, to the entire length of the nucleotide sequence) shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, or a complement thereof.

In a preferred embodiment, the isolated nucleic acid molecule includes the nucleotide sequence shown SEQ ID NO:1 or 3, or a complement thereof. In another embodiment, the nucleic acid molecule includes SEQ ID NO:3 and nucleotides 1-9 of SEQ

ID NO:1. In yet another embodiment, the nucleic acid molecule includes SEQ ID NO:3 and nucleotides 1507-3452 of SEQ ID NO:1. In another preferred embodiment, the nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:1 or 3. In another preferred embodiment, the nucleic acid molecule includes a fragment of at least 1644 nucleotides of the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, or a complement thereof.

In another preferred embodiment, the isolated nucleic acid molecule includes the nucleotide sequence shown SEQ ID NO:4 or 6, or a complement thereof. In another embodiment, the nucleic acid molecule includes SEQ ID NO:6 and nucleotides 1-121 of SEQ ID NO:4. In yet another embodiment, the nucleic acid molecule includes SEQ ID NO:6 and nucleotides 1118-1575 of SEQ ID NO:4. In another preferred embodiment, the nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:4 or 6. In another preferred embodiment, the nucleic acid molecule includes a fragment of at least 369 nucleotides of the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:6, or a complement thereof.

In another preferred embodiment, the isolated nucleic acid molecule includes the nucleotide sequence shown SEQ ID NO:7 or 9, or a complement thereof. In another embodiment, the nucleic acid molecule includes SEQ ID NO:9 and nucleotides 1-135 of SEQ ID NO:7. In yet another embodiment, the nucleic acid molecule includes SEQ ID NO:9 and nucleotides 1075-2287 of SEQ ID NO:7. In another preferred embodiment, the nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:7 or 9. In another preferred embodiment, the nucleic acid molecule includes a fragment of at least 537 nucleotides of the nucleotide sequence of SEQ ID NO:7, SEQ ID NO:9, or a complement thereof.

In yet another preferred embodiment, the isolated nucleic acid molecule includes the nucleotide sequence shown SEQ ID NO:10 or 12, or a complement thereof. In another embodiment, the nucleic acid molecule includes SEQ ID NO:12 and nucleotides 1-156 of SEQ ID NO:10. In yet another embodiment, the nucleic acid molecule includes SEQ ID NO:12 and nucleotides 1361-1506 of SEQ ID NO:10. In another preferred embodiment, the nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:10 or 12.

In another embodiment, a TWIK nucleic acid molecule includes a nucleotide sequence encoding a protein having an amino acid sequence sufficiently identical to the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:11, or an amino acid sequence encoded by the DNA insert of the plasmid deposited with ATCC as Accession Number _____ . In a preferred embodiment, a TWIK nucleic acid molecule includes a nucleotide sequence encoding a protein having an amino acid sequence at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more identical to the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8,

or SEQ ID NO:11, or the amino acid sequence encoded by the DNA insert of the plasmid deposited with ATCC as Accession Number _____.

In another preferred embodiment, an isolated nucleic acid molecule encodes the amino acid sequence of human TWIK-2, TWIK-3, TWIK-4, or TWIK-5. In yet another preferred embodiment, the nucleic acid molecule includes a nucleotide sequence encoding a protein having the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, or SEQ ID NO:11, or the amino acid sequence encoded by the DNA insert of the plasmid deposited with ATCC as Accession Number _____. In yet another preferred embodiment, the nucleic acid molecule is at least 537 nucleotides in length and encodes a protein having a TWIK activity (as described herein).

Another embodiment of the invention features nucleic acid molecules, preferably TWIK nucleic acid molecules, which specifically detect TWIK nucleic acid molecules relative to nucleic acid molecules encoding non-TWIK proteins. For example, in one embodiment, such a nucleic acid molecule is at least 369, 400-450, 450-500, 500-550, 550-600, 600-650, 650-700, 700-750, 750-800 or more nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence shown in SEQ ID NO:4, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number_____, or a complement thereof. In preferred embodiments, the nucleic acid molecules are at least 15 (*e.g.*, contiguous) nucleotides in length and hybridize under stringent conditions to nucleotides 1-397, 586-670, 904-1111, or 1573-1575 of SEQ ID NO:4. In other preferred embodiments, the nucleic acid molecules comprise nucleotides 1-397, 586-670, 904-1111, or 1573-1575 of SEQ ID NO:4.

In another particularly preferred embodiment, the nucleic acid molecule comprises a fragment of at least 537, 550-600, 600-650, 650-700, 700-750, 750-800 or more nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence shown in SEQ ID NO:7, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number_____, or a complement thereof. In preferred embodiments, the nucleic acid molecules are at least 15 (*e.g.*, contiguous) nucleotides in length and hybridize under stringent conditions to nucleotides 1-164, 207-404, 1037-1789, 1818-1869, 1972-1985, or 2258-2287 of SEQ ID NO:7. In other preferred embodiments, the nucleic acid molecules include nucleotides 1-164, 207-404, 1037-1789, 1818-1869, 1972-1985, or 2258-2287 of SEQ ID NO:7.

In another particularly preferred embodiment, the nucleic acid molecule comprises a fragment of at least 550-600, 600-650, 650-700, 700-750, 750-800, 805, 850-900 or more nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence shown in SEQ ID NO:10, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number_____, or a complement thereof.

In other preferred embodiments, the nucleic acid molecule encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, 5, 8, 11, or an amino acid sequence encoded by the DNA insert of the plasmid deposited with ATCC as Accession Number _____, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:1, 3, 4, 6, 7, 9, 10, or 12 under stringent conditions.

Another embodiment of the invention provides an isolated nucleic acid molecule which is antisense to a TWIK nucleic acid molecule, *e.g.*, the coding strand of a TWIK nucleic acid molecule.

Another aspect of the invention provides a vector comprising a TWIK nucleic acid molecule. In certain embodiments, the vector is a recombinant expression vector. In another embodiment, the invention provides a host cell containing a vector of the invention. The invention also provides a method for producing a protein, preferably a TWIK protein, by culturing in a suitable medium, a host cell, *e.g.*, a mammalian host cell such as a non-human mammalian cell, of the invention containing a recombinant expression vector, such that the protein is produced.

Another aspect of this invention features isolated or recombinant TWIK proteins and polypeptides. In one embodiment, the isolated protein, preferably a TWIK protein, includes at least one transmembrane domain. In another embodiment, the isolated protein, preferably a TWIK protein, includes at least one P-loop. In another embodiment, the isolated protein, preferably a TWIK protein, includes at least one transmembrane domain and at least one P-loop. In a preferred embodiment, the protein, preferably a TWIK protein, includes at least one transmembrane domain and at least one P-loop and has an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more identical to the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:11 or the amino acid sequence encoded by the DNA insert of the plasmid deposited with ATCC as Accession Number _____. In another preferred embodiment, the protein, preferably a TWIK protein, includes at least one transmembrane domain and plays a role in generating an electrical potential across a plasma membrane, *e.g.*, a neuronal plasma membrane or a muscle plasma membrane. In another preferred embodiment, the protein, preferably a TWIK protein, includes at least one P-loop and plays a role in generating an electrical potential across a plasma membrane, *e.g.*, a neuronal plasma membrane or a muscle plasma membrane. In another preferred embodiment, the protein, preferably a TWIK protein, includes at least one transmembrane domain and at least one P-loop, and plays a role in generating an electrical potential across a plasma membrane, *e.g.*, a neuronal plasma membrane or a muscle plasma membrane. In yet another preferred embodiment, the protein, preferably a TWIK protein, includes at least one transmembrane domain and at least one P-loop and is encoded by a nucleic acid molecule having a nucleotide sequence which

hybridizes under stringent hybridization conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:10, or SEQ ID NO:12.

In another embodiment, the invention features fragments of the proteins having the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, or SEQ ID NO:11, wherein the fragment comprises at least 15 amino acids (*e.g.*, contiguous amino acids) of the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11, or an amino acid sequence encoded by the DNA insert of the plasmid deposited with the ATCC as Accession Number_____ or _____. In another embodiment, the protein, preferably a TWIK protein, has the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11.

In another embodiment, the invention features an isolated protein, preferably a TWIK protein, which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more identical to a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:12, or a complement thereof.

This invention further features an isolated protein, preferably a TWIK protein, which is encoded by a nucleic acid molecule having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or a complement thereof.

The proteins of the present invention or biologically active portions thereof, can be operatively linked to a non-TWIK polypeptide (*e.g.*, heterologous amino acid sequences) to form fusion proteins. The invention further features antibodies, such as monoclonal or polyclonal antibodies, that specifically bind proteins of the invention, preferably TWIK proteins. In addition, the TWIK proteins or biologically active portions thereof can be incorporated into pharmaceutical compositions, which optionally include pharmaceutically acceptable carriers.

In another aspect, the present invention provides a method for detecting the presence of a TWIK nucleic acid molecule, protein or polypeptide in a biological sample by contacting the biological sample with an agent capable of detecting a TWIK nucleic acid molecule, protein or polypeptide such that the presence of a TWIK nucleic acid molecule, protein or polypeptide is detected in the biological sample.

In another aspect, the present invention provides a method for detecting the presence of TWIK activity in a biological sample by contacting the biological sample with an agent capable of detecting an indicator of TWIK activity such that the presence of TWIK activity is detected in the biological sample.

In another aspect, the invention provides a method for modulating TWIK activity comprising contacting a cell capable of expressing TWIK with an agent that modulates TWIK activity such that TWIK activity in the cell is modulated. In one embodiment, the agent inhibits TWIK activity. In another embodiment, the agent stimulates TWIK activity. In one embodiment, the agent is an antibody that specifically binds to a TWIK protein. In another embodiment, the agent modulates expression of TWIK by modulating transcription of a TWIK gene or translation of a TWIK mRNA. In yet another embodiment, the agent is a nucleic acid molecule having a nucleotide sequence that is antisense to the coding strand of a TWIK mRNA or a TWIK gene.

In one embodiment, the methods of the present invention are used to treat a subject having a disorder characterized by aberrant TWIK protein or nucleic acid expression or activity by administering an agent which is a TWIK modulator to the subject. In one embodiment, the TWIK modulator is a TWIK protein. In another embodiment the TWIK modulator is a TWIK nucleic acid molecule. In yet another embodiment, the TWIK modulator is a peptide, peptidomimetic, or other small molecule. In a preferred embodiment, the disorder characterized by aberrant TWIK protein or nucleic acid expression is a CNS disorder.

The present invention also provides a diagnostic assay for identifying the presence or absence of a genetic alteration characterized by at least one of (i) aberrant modification or mutation of a gene encoding a TWIK protein; (ii) mis-regulation of the gene; and (iii) aberrant post-translational modification of a TWIK protein, wherein a wild-type form of the gene encodes a protein with a TWIK activity.

In another aspect the invention provides a method for identifying a compound that binds to or modulates the activity of a TWIK protein, by providing an indicator composition comprising a TWIK protein having TWIK activity, contacting the indicator composition with a test compound, and determining the effect of the test compound on TWIK activity in the indicator composition to identify a compound that modulates the activity of a TWIK protein.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

Figure 1 depicts the cDNA sequence and predicted amino acid sequence of human TWIK-2. The nucleotide sequence corresponds to nucleic acids 1 to 3452 of SEQ ID NO:1. The amino acid sequence corresponds to amino acids 1 to 499 of SEQ ID NO:2. The coding region without the 5' and 3' untranslated regions of the human TWIK-2 gene is shown in SEQ ID NO:3.

Figure 2 depicts a structural, hydrophobicity, and antigenicity analysis of the human TWIK-2 protein.

Figure 3 depicts the cDNA sequence and predicted amino acid sequence of human TWIK-3. The nucleotide sequence corresponds to nucleic acids 1 to 1575 of SEQ ID NO:4. The amino acid sequence corresponds to amino acids 1 to 332 of SEQ ID NO:5. The coding region without the 5' and 3' untranslated regions of the human TWIK-3 gene is shown in SEQ ID NO:6.

Figure 4 depicts a structural, hydrophobicity, and antigenicity analysis of the human TWIK-3 protein.

Figure 5 depicts the cDNA sequence and predicted amino acid sequence of human TWIK-4. The nucleotide sequence corresponds to nucleic acids 1 to 2287 of SEQ ID NO:7. The amino acid sequence corresponds to amino acids 1 to 313 of SEQ ID NO:8. The coding region without the 5' and 3' untranslated regions of the human TWIK-4 gene is shown in SEQ ID NO:9.

Figure 6 depicts a structural, hydrophobicity, and antigenicity analysis of the human TWIK-4 protein.

Figure 7 depicts a multiple sequence alignment of the amino acid sequence of human TWIK-4, human TWIK-1, murine TRAAK, murine TREK-1, human TWIK-3, human TWIK-2, and human TASK.

Figure 8 depicts a multiple sequence alignment of the amino acid sequence of human TWIK-1, human TWIK-4, murine TRAAK, murine TREK-1, and human TASK.

Figure 9 depicts a multiple sequence alignment of the amino acid sequence of human TWIK-3, human TASK2, human TWIK-1, and human TASK.

Figure 10 depicts an alignment of the TWIK-2 protein with the TWIK-3 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4.

Figure 11 depicts an alignment of the TWIK-2 protein with the TWIK-4 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4.

Figure 12 depicts an alignment of the TWIK-3 protein with the TWIK-4 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4.

Figure 13 depicts an alignment of the TWIK-1 protein with the TWIK-2 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4.

Figure 14 depicts an alignment of the TWIK-1 protein with the TWIK-3 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4.

Figure 15 depicts an alignment of the TWIK-1 protein with the TWIK-4 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4.

Figure 16 depicts an alignment of the hTASK-2 protein with the TWIK-2 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4.

Figure 17 depicts the cDNA sequence and predicted amino acid sequence of human TWIK-5. The nucleotide sequence corresponds to nucleic acids 1 to 1506 of SEQ ID NO:10. The amino acid sequence corresponds to amino acids 1 to 401 of SEQ ID NO:11. The coding region without the 5' and 3' untranslated regions of the human TWIK-5 gene is shown in SEQ ID NO:12.

Figure 18 depicts a structural, hydrophobicity, and antigenicity analysis of the human TWIK-5 protein.

Figure 19 depicts an alignment of the TWIK-5 protein with the hTASK-2 protein using the GAP program in the GCG software package (PAM250 matrix) and a gap weight of 25 and a length weight of 1.

Figure 20 depicts an alignment of the TWIK-5 protein with the mouse TREK protein using the GAP program in the GCG software package (PAM250 matrix) and a gap weight of 25 and a length weight of 1.

Figure 21 depicts a recording of the outward currents of TWIK-5 channels in transiently transfected CHO cells recorded under whole-cell patch-clamp. Panel A, lower portion depicts a schematic of the electrophysiological protocol for the experiment (described in Example 4), and the upper portion of Panel A depicts the results of the experiment. Panel B is a graph depicting the relationship between current and membrane potential in TWIK-5 channel activation.

Figure 22 is a graphical depiction of the results of experiments designed to assess the pharmacological properties of the TWIK-5 channel. The outward currents of TWIK-5 channels in transiently transfected CHO cells under whole-cell patch clamp are shown in Panel A; Panels B-D show similar recordings taken in the presence of 10 mM Ba^{++}, 10 mM triethylammonium, and 10 mM 4-aminopyridine, respectively.

Detailed Description of the Invention

The present invention is based, at least in part, on the discovery of novel molecules, referred to herein as TWIK-2, TWIK-3, TWIK-4, and TWIK-5 nucleic acid and protein molecules, which are novel members of the TWIK (for **T**andem of P domains in a **W**eak **I**nward rectifying $\mathbf{K}^+$ channel) family of potassium channels. These novel molecules are capable of, for example, modulating a potassium channel mediated activity in a cell, *e.g.*, a neuronal cell or a muscle cell.

As used herein, a "potassium channel" includes a protein or polypeptide which is involved in receiving, conducting, and transmitting signals, in an electrically excitable cell, *e.g.*, a neuronal cell or a muscle cell. Potassium channels are potassium ion selective, and can determine membrane excitability (the ability of, for example, a neuron to respond to a stimulus and convert it into an impulse). Potassium channels can also influence the resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation. Potassium channels are typically expressed in electrically excitable cells, *e.g.*, neurons, muscle, endocrine, and egg cells, and may form heteromultimeric structures, *e.g.*, composed of pore-forming α and cytoplasmic β subunits. Examples of potassium channels include: (1) the voltage-gated potassium channels, (2) the ligand-gated potassium channels, *e.g.*, cyclic nucleotide-gated potassium channels, and (3) the mechanically-gated potassium channels. Voltage-gated and ligand-gated potassium channels are expressed in the brain, *e.g.*, in brainstem monoaminergic and forebrain cholinergic neurons, where they are involved in the release of neurotransmitters, or in the dendrites of hippocampal and neocortical pyramidal cells, where they are involved in the processes of learning and memory formation. For a detailed description of potassium channels, see Kandel E.R.. *et al.*, Principles of Neural Science, second edition, (Elsevier Science Publishing Co., Inc., N.Y. (1985)), the contents of which are incorporated herein by reference. As the TWIK proteins of the present invention may modulate potassium channel mediated activities, they may be useful for developing novel diagnostic and therapeutic agents for potassium channel associated disorders.

As used herein, a "potassium channel associated disorder" includes a disorder, disease or condition which is characterized by a misregulation of a potassium channel mediated activity. Potassium channel associated disorders can detrimentally affect conveyance of sensory impulses from the periphery to the brain and/or conductance of motor impulses from the brain to the periphery; integration of reflexes; interpretation of sensory impulses; and emotional, intellectual (*e.g.*, learning and memory), or motor processes. Examples of potassium channel associated disorders include CNS disorders such as neurodegenerative disorders, *e.g.*, Alzheimer's disease, dementias related to Alzheimer's disease (such as Pick's disease), Parkinson's and other Lewy diffuse body diseases, multiple sclerosis, amyotrophic lateral sclerosis, progressive supranuclear palsy, epilepsy, and Jakob-Creutzfieldt disease; psychiatric disorders, *e.g.*, depression, schizophrenic disorders, korsakoff's psychosis, mania, anxiety disorders, or phobic disorders; learning or memory disorders, *e.g.*, amnesia or age-related memory loss; and neurological disorders, *e.g.*, migraine. Further examples of potassium channel associated disorders include obesity; cardiac disorders, *e.g.*, cardiac arrythmias; and pain disorders, *e.g.*, pain disorders associated with various forms of tissue injury, such as inflammation, infection, and ischemia, usually referred to as hyperalgesia (described in, for example, Fields, H.L. (1987) *Pain*, New

York:McGraw-Hill), tooth pain, headaches (*e.g.*, tension headache or migraine), back pain, cancer pain, arthritis pain, psychogenic pain, pain associated with surgery, or neuropathic pain.

As used herein, a "potassium channel mediated activity" includes an activity which involves a potassium channel, *e.g.*, a potassium channel in a neuronal cell or a muscle cell, associated with receiving, conducting, and transmitting signals in, for example, the nervous system. Potassium channel mediated activities include release of neurotransmitters, *e.g.*, dopamine or norepinephrine, from cells, *e.g.*, neuronal cells; modulation of resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation; and modulation of processes such as integration of sub-threshold synaptic responses and the conductance of back-propagating action potentials in, for example, neuronal cells or muscle cells.

The term "family" when referring to the protein and nucleic acid molecules of the invention is intended to mean two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide sequence homology as defined herein. Such family members can be naturally or non-naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or alternatively, can contain homologues of non-human origin. Members of a family may also have common functional characteristics.

For example, the family of TWIK proteins comprise at least one "transmembrane domain" and preferably four transmembrane domains. As used herein, the term "transmembrane domain" includes an amino acid sequence of about 15 amino acid residues in length which spans the plasma membrane. More preferably, a transmembrane domain includes about at least 20, 25, 30, 35, 40, or 45 amino acid residues and spans the plasma membrane. Transmembrane domains are rich in hydrophobic residues, and typically have an α-helical structure. In a preferred embodiment, at least 50%, 60%, 70%, 80%, 90%, 95% or more of the amino acids of a transmembrane domain are hydrophobic, *e.g.*, leucines, isoleucines, tyrosines, or tryptophans. Transmembrane domains are described in, for example, Zagotta W.N. et al, (1996) *Annual Rev. Neuronsci.* 19: 235-63, the contents of which are incorporated herein by reference. Amino acid residues 7-23, 113-134, 160-184, and 225-242 of the human TWIK-2 protein, amino acid residues 23-43, 131-148, 178-200, and 244-264 of the human TWIK-3 protein, amino acid residues 7-26, 121-140, 169-193, and 236-253 of the human TWIK-4 protein comprise transmembrane domains, and amino acid residues 37-61, 148-165, 298-321, and 353-372 of the human TWIK-5 protein comprise transmembrane domains.

In another embodiment, a TWIK molecule of the present invention is identified based on the presence of a P-loop. As used herein, the term "P-loop" (also known as an H5

domain) includes an amino acid sequence of about 15-45 amino acid residues in length, preferably about 15-35 amino acid residues in length, and most preferably about 15-25 amino acid residues in length, which is involved in lining the potassium channel pore. A P-loop is typically found between transmembrane domains of potassium channels and is believed to be a major determinant of ion selectivity in potassium channels. Preferably, P-loops contain a G-[HYDROPHOBIC AMINO ACID]-G sequence, *e.g.*, a GYG, GLG, or GFG sequence. P-loops are described in, for example, Warmke *et al.* (1991) *Science* 252:1560-1562; Zagotta W.N. *et al.*, (1996) *Annual Rev. Neuronsci.* 19:235-63 (Pongs, O. (1993) *J. Membr. Biol.*, 136, 1-8; Heginbotham *et al.* (1994) *Biophys. J.* 66,1061-1067; Mackinnon, R. (1995) *Neuron*, and 14, 889-892; Pascual *et al.*, (1995) *Neuron.*, 14, 1055-1063), the contents of which are incorporated herein by reference. Amino acid residues 88-105 and 194-213 of the human TWIK-2 protein, amino acid residues 103-119 and 212-229 of the human TWIK-3 protein, amino acid residues 93-109 and 204-221 of the human TWIK-4 protein comprise a P-loop, and amino acid residues 118-139 and 328-345 of the human TWIK-5 protein comprise a P-loop.

In a preferred embodiment, the TWIK molecules of the invention include an intracellular amino- and carboxyl- terminus, four transmembrane domains, and two P-loops. The P-loops are preferably located between transmembrane domains 1 and 2, and 3 and 4. The TWIK molecules of the present invention can further include a cysteine residue between the first transmembrane domain and the first P-loop, which may be involved in the formation of a TWIK dimer (through a disulfide bridge). For example, TWIK-2 contains such a cysteine residue at position 51, TWIK-3 contains such a cysteine residue at position 68, TWIK-4 contains such a cysteine residue at position 53, and TWIK-5 contains such a cysteine residue at position 83.

Isolated proteins of the present invention, preferably TWIK-2, TWIK-3, TWIK-4, and TWIK-5 proteins, have an amino acid sequence sufficiently identical to the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:11 or are encoded by a nucleotide sequence sufficiently identical to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:12. As used herein, the term "sufficiently identical" refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent (*e.g.*, an amino acid residue which has a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences share common structural domains or motifs and/or a common functional activity. For example, amino acid or nucleotide sequences which share common structural domains have at least 30%, 40%, or 50% homology, preferably 60% homology, more preferably 70%-80%, and even more preferably 90-95% homology across the amino acid sequences of the domains and contain at least one and preferably two structural domains or motifs, are defined herein as sufficiently

identical. Furthermore, amino acid or nucleotide sequences which share at least 30%, 40%, or 50%, preferably 60%, more preferably 70-80%, or 90-95% homology and share a common functional activity are defined herein as sufficiently identical.

As used interchangeably herein, a "TWIK activity", "biological activity of TWIK" or "functional activity of TWIK", refers to an activity exerted by a TWIK protein, polypeptide or nucleic acid molecule on a TWIK responsive cell or on a TWIK protein substrate, as determined *in vivo*, or *in vitro*, according to standard techniques. In one embodiment, a TWIK activity is a direct activity, such as an association with a TWIK-target molecule. As used herein, a "target molecule" or "binding partner" is a molecule with which a TWIK protein binds or interacts in nature, such that TWIK-mediated function is achieved. A TWIK target molecule can be a non-TWIK molecule or a TWIK protein or polypeptide of the present invention. In an exemplary embodiment, a TWIK target molecule is a TWIK ligand. Alternatively, a TWIK activity is an indirect activity, such as a cellular signaling activity mediated by interaction of the TWIK protein with a TWIK ligand. The biological activities of TWIK are described herein. For example, the TWIK proteins of the present invention can have one or more of the following activities: (1) modulate the release of neurotransmitters, (2) modulate membrane excitability, (3) influence the resting potential of membranes, (4) modulate wave forms and frequencies of action potentials, (5) modulate thresholds of excitation, and (6) modulate processes which underlie learning and memory, such as integration of sub-threshold synaptic responses and the conductance of back-propagating action potentials.

Accordingly, another embodiment of the invention features isolated TWIK proteins and polypeptides having a TWIK activity. Preferred proteins are TWIK proteins having at least one transmembrane domain, at least one P-loop and, preferably, a TWIK activity. Other preferred proteins are TWIK proteins having at least one transmembrane domain and, preferably, a TWIK activity. Other preferred proteins are TWIK proteins having at least one P-loop, and, preferably, a TWIK activity. Other preferred proteins are TWIK proteins having at least one transmembrane domain, at least one P-loop, and are, preferably, encoded by a nucleic acid molecule having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:12.

The nucleotide sequence of the isolated human TWIK-2 cDNA and the predicted amino acid sequence of the human TWIK-2 polypeptide are shown in Figure 1 and in SEQ ID NOs:1 and 2, respectively. A plasmid containing the nucleotide sequence encoding human TWIK-2 was deposited with American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, on _____ and assigned Accession Number _____. This deposit will be maintained under the terms of the Budapest Treaty on

the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

The human TWIK-2 gene, which is approximately 3452 nucleotides in length, encodes a protein having a molecular weight of approximately 57.4 kD and which is approximately 499 amino acid residues in length.

The nucleotide sequence of the isolated human TWIK-3 cDNA and the predicted amino acid sequence of the human TWIK-3 polypeptide are shown in Figure 3 and in SEQ ID NOs:4 and 5, respectively. A plasmid containing the nucleotide sequence encoding human TWIK-3 was deposited with American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, on _____ and assigned Accession Number _____. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

The human TWIK-3 gene, which is approximately 1575 nucleotides in length, encodes a protein having a molecular weight of approximately 38.2 kD and which is approximately 332 amino acid residues in length.

The nucleotide sequence of the isolated human TWIK-4 cDNA and the predicted amino acid sequence of the human TWIK-4 polypeptide are shown in Figure 5 and in SEQ ID NOs:7 and 9, respectively. A plasmid containing the nucleotide sequence encoding human TWIK-4 was deposited with American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, on _____ and assigned Accession Number _____. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

The human TWIK-4 gene, which is approximately 2287 nucleotides in length, encodes a protein having a molecular weight of approximately 36 kD and which is approximately 313 amino acid residues in length.

The nucleotide sequence of the isolated human TWIK-5 cDNA and the predicted amino acid sequence of the human TWIK-5 polypeptide are shown in Figure 17 and in SEQ ID NOs:10 and 11, respectively.

The human TWIK-5 gene, which is approximately 1506 nucleotides in length, encodes a protein having a molecular weight of approximately 46.1 kD and which is approximately 401 amino acid residues in length.

Various aspects of the invention are described in further detail in the following subsections:

I. Isolated Nucleic Acid Molecules

One aspect of the invention pertains to isolated nucleic acid molecules that encode TWIK proteins or biologically active portions thereof, as well as nucleic acid fragments sufficient for use as hybridization probes to identify TWIK-encoding nucleic acid molecules (*e.g.*, TWIK mRNA) and fragments for use as PCR primers for the amplification or mutation of TWIK nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated TWIK nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or portion of the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, as a hybridization probe, TWIK nucleic acid molecules can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____.

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to TWIK nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1. The sequence of SEQ ID NO:1 corresponds to the human TWIK-2 cDNA. This cDNA comprises sequences encoding the human TWIK-2 protein (*i.e.*, "the coding region", from nucleotides 10-1506), as well as 5' untranslated sequences (nucleotides 1-9) and 3' untranslated sequences (nucleotides 1507-3452). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:1 (*e.g.*, nucleotides 10-1506, corresponding to SEQ ID NO:3).

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:4. The sequence of SEQ ID NO:4 corresponds to the human TWIK-3 cDNA. This cDNA comprises sequences encoding the human TWIK-3 protein (*i.e.*, "the coding region", from nucleotides 122-1117), as well as 5' untranslated sequences (nucleotides 1-121) and 3' untranslated sequences (nucleotides 1118-1575). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:4 (*e.g.*, nucleotides 122-1117, corresponding to SEQ ID NO:6).

In yet another preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:7. The sequence of SEQ ID NO:7 corresponds to the human TWIK-4 cDNA. This cDNA comprises sequences encoding the human TWIK-4 protein (*i.e.*, "the coding region", from nucleotides 136-1074), as well as 5' untranslated sequences (nucleotides 1-135) and 3' untranslated sequences (nucleotides 1075-2287). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:7 (*e.g.*, nucleotides 136-1074, corresponding to SEQ ID NO:9).

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:10. The sequence of SEQ ID

NO:10 corresponds to the human TWIK-5 cDNA. This cDNA comprises sequences encoding the human TWIK-5 protein (*i.e.*, "the coding region", from nucleotides 157-1359), as well as 5' untranslated sequences (nucleotides 1-156) and 3' untranslated sequences (nucleotides 1360-1506). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:10 (*e.g.*, nucleotides 157-1359, corresponding to SEQ ID NO:12).

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or ____, or a portion of any of these nucleotide sequences. A nucleic acid molecule which is complementary to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____or _____, is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____or _____, such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, thereby forming a stable duplex.

In still another preferred embodiment, an isolated nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more identical to the entire length of the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the entire length of the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, or a portion of any of these nucleotide sequences.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____or _____, for example a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of a TWIK protein. The nucleotide sequence determined from the cloning of the TWIK gene allows for the generation of probes and

primers designed for use in identifying and/or cloning other TWIK family members, as well as TWIK homologues from other species.

The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12 or 15, preferably about 20 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 consecutive nucleotides of a sense sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____or _____, of an anti-sense sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____or _____, or of a naturally occurring allelic variant or mutant of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____. In an exemplary embodiment, a nucleic acid molecule of the present invention comprises a nucleotide sequence which is 369, 350-400, 400-450, 450-500, 500-550, 537, 550-600, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 949, 950-1000, 1575, 2287, or 3452 nucleotides in length and hybridizes under stringent hybridization conditions to a nucleic acid molecule of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____.

Probes based on the TWIK nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or identical proteins. In preferred embodiments, the probe further comprises a label group attached thereto, *e.g.*, the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a TWIK protein, such as by measuring a level of a TWIK-encoding nucleic acid in a sample of cells from a subject *e.g.*, detecting TWIK mRNA levels or determining whether a genomic TWIK gene has been mutated or deleted.

A nucleic acid fragment encoding a "biologically active portion of a TWIK protein" can be prepared by isolating a portion of the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, which encodes a polypeptide having a TWIK biological activity (the biological activities of the TWIK proteins are described herein),

expressing the encoded portion of the TWIK protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the TWIK protein.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12 or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, due to degeneracy of the genetic code and thus encode the same TWIK proteins as those encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12 or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____. In another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11.

In addition to the TWIK nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the TWIK proteins may exist within a population (*e.g.*, the human population). Such genetic polymorphism in the TWIK genes may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which include an open reading frame encoding a TWIK protein, preferably a mammalian TWIK protein, and can further include non-coding regulatory sequences, and introns.

Allelic variants of human TWIK include both functional and non-functional TWIK proteins. Functional allelic variants are naturally occurring amino acid sequence variants of the human TWIK protein that maintain the ability to bind a TWIK ligand and/or modulate any of the TWIK activities described herein. Functional allelic variants will typically contain only conservative substitution of one or more amino acids of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11 or substitution, deletion or insertion of non-critical residues in non-critical regions of the protein.

Non-functional allelic variants are naturally occurring amino acid sequence variants of the human TWIK protein that do not have the ability to either bind a TWIK ligand and/or modulate any of the TWIK activities described herein. Non-functional allelic variants will typically contain a non-conservative substitution, a deletion, or insertion or premature truncation of the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11 or a substitution, insertion or deletion in critical residues or critical regions.

The present invention further provides non-human orthologues of the human TWIK protein. Orthologues of the human TWIK protein are proteins that are isolated from non-human organisms and possess the same TWIK ligand binding and/or potassium channel mediated activities of the human TWIK protein. Orthologues of the human TWIK protein can readily be identified as comprising an amino acid sequence that is substantially identical to SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11.

Moreover, nucleic acid molecules encoding other TWIK family members and, thus, which have a nucleotide sequence which differs from the TWIK sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____ are intended to be within the scope of the invention. For example, another TWIK cDNA can be identified based on the nucleotide sequence of human TWIK. Moreover, nucleic acid molecules encoding TWIK proteins from different species, and thus which have a nucleotide sequence which differs from the TWIK sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12 or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____ are intended to be within the scope of the invention. For example, a mouse TWIK cDNA can be identified based on the nucleotide sequence of a human TWIK.

Nucleic acid molecules corresponding to natural allelic variants and homologues of the TWIK cDNAs of the invention can be isolated based on their homology to the TWIK nucleic acids disclosed herein using the cDNAs disclosed herein, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 15, 20, 25, 30 or more nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12 or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____. In other embodiment, the nucleic acid is at least 30, 50, 100, 150, 200, 250, 300, 307, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 949, or 950 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% identical to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in*

Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50°C, preferably at 55°C, and more preferably at 60°C or 65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1 corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

In addition to naturally-occurring allelic variants of the TWIK sequences that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, thereby leading to changes in the amino acid sequence of the encoded TWIK proteins, without altering the functional ability of the TWIK proteins. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of TWIK (*e.g.*, the sequence of SEQ ID NO:2) without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the TWIK proteins of the present invention, are predicted to be particularly unamenable to alteration. Furthermore, additional amino acid residues that are conserved between the TWIK proteins of the present invention and other members of the TWIK potassium channel families are not likely to be amenable to alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding TWIK proteins that contain changes in amino acid residues that are not essential for activity. Such TWIK proteins differ in amino acid sequence from SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11, yet retain biological activity. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more identical to SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11.

An isolated nucleic acid molecule encoding a TWIK protein identical to the protein of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide

sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____ by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a TWIK protein is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a TWIK coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for TWIK biological activity to identify mutants that retain activity. Following mutagenesis of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

In a preferred embodiment, a mutant TWIK protein can be assayed for the ability to (1) interact with a non-TWIK protein molecule; (2) activate a TWIK-dependent signal transduction pathway; (3) modulate the release of neurotransmitters, (4) modulate membrane excitability, (5) influence the resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation, and (6) modulate processes which underlie learning and memory, such as integration of sub-threshold synaptic responses and the conductance of back-propagating action potentials.

In addition to the nucleic acid molecules encoding TWIK proteins described above, another aspect of the invention pertains to isolated nucleic acid molecules which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the

coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire TWIK coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding TWIK. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues (*e.g.*, the coding region of human TWIK-2 corresponds to SEQ ID NO:3). In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding TWIK. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

Given the coding strand sequences encoding TWIK disclosed herein (*e.g.*, SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, and SEQ ID NO:12), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of TWIK mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of TWIK mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of TWIK mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-

N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a TWIK protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids. Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave TWIK mRNA transcripts to thereby inhibit translation of TWIK mRNA. A ribozyme having specificity for a TWIK-encoding nucleic acid can be designed based upon the nucleotide sequence of a TWIK cDNA disclosed herein (*i.e.*, SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID

NO:12, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a TWIK-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, TWIK mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

Alternatively, TWIK gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the TWIK (*e.g.*, the TWIK promoter and/or enhancers) to form triple helical structures that prevent transcription of the TWIK gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioassays* 14(12):807-15.

In yet another embodiment, the TWIK nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup B. *et al.* (1996) *Bioorganic & Medicinal Chemistry* 4 (1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup B. *et al.* (1996) *supra*; Perry-O'Keefe *et al. Proc. Natl. Acad. Sci.* 93: 14670-675.

PNAs of TWIK nucleic acid molecules can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of TWIK nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as 'artificial restriction enzymes' when used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup B. (1996) *supra*)); or as probes or primers for DNA sequencing or hybridization (Hyrup B. *et al.* (1996) *supra*; Perry-O'Keefe *supra*).

In another embodiment, PNAs of TWIK can be modified, (*e.g.*, to enhance their stability or cellular uptake), by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of TWIK nucleic acid

molecules can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, (*e.g.*, RNAse H and DNA polymerases), to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup B. (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup B. (1996) *supra* and Finn P.J. *et al.* (1996) *Nucleic Acids Res.* 24 (17): 3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, *e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag, M. *et al.* (1989) *Nucleic Acid Res.* 17: 5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn P.J. *et al.* (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K.H. *et al.* (1975) *Bioorganic Med. Chem. Lett.* 5: 1119-11124).

In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. US.* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.* (1988) *Bio-Techniques* 6:958-976) or intercalating agents. (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

II. Isolated TWIK Proteins and Anti-TWIK Antibodies

One aspect of the invention pertains to isolated TWIK proteins, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise anti-TWIK antibodies. In one embodiment, native TWIK proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, TWIK proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a TWIK protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the TWIK protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially

free of cellular material" includes preparations of TWIK protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of TWIK protein having less than about 30% (by dry weight) of non-TWIK protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-TWIK protein, still more preferably less than about 10% of non-TWIK protein, and most preferably less than about 5% non-TWIK protein. When the TWIK protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of TWIK protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of TWIK protein having less than about 30% (by dry weight) of chemical precursors or non-TWIK chemicals, more preferably less than about 20% chemical precursors or non-TWIK chemicals, still more preferably less than about 10% chemical precursors or non-TWIK chemicals, and most preferably less than about 5% chemical precursors or non-TWIK chemicals.

As used herein, a "biologically active portion" of a TWIK protein includes a fragment of a TWIK protein which participates in an interaction between a TWIK molecule and a non-TWIK molecule. Biologically active portions of a TWIK protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the TWIK protein, *e.g.*, the amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11, which include less amino acids than the full length TWIK proteins, and exhibit at least one activity of a TWIK protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the TWIK protein, *e.g.*, binding of a cyclic nucleotide. A biologically active portion of a TWIK protein can be a polypeptide which is, for example, 10, 25, 50, 100, 200, 313, 332, or 499 amino acids in length. Biologically active portions of a TWIK protein can be used as targets for developing agents which modulate a potassium channel mediated activity.

In one embodiment, a biologically active portion of a TWIK protein comprises at least one transmembrane domain. In another embodiment, a biologically active portion of a TWIK protein comprises at least one P-loop. In yet another embodiment a biologically active portion of a TWIK protein comprises at least one transmembrane domain and at least one P-loop.

It is to be understood that a preferred biologically active portion of a TWIK protein of the present invention may contain at least one of the above-identified structural domains. A more preferred biologically active portion of a TWIK protein may contain at least two of the above-identified structural domains. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native TWIK protein.

In a preferred embodiment, the TWIK protein has an amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11. In other embodiments, the TWIK protein is substantially homologous to SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11, and retains the functional activity of the protein of SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11, yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail in subsection I above. Accordingly, in another embodiment, the TWIK protein is a protein which comprises an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more identical to SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (*e.g.*, when aligning a second sequence to the TWIK amino acid sequence of SEQ ID NO:2, 5, or 8 having 177 amino acid residues, at least 80, preferably at least 100, more preferably at least 120, even more preferably at least 140, and even more preferably at least 150, 160 or 170 amino acid residues are aligned). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into

the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to TWIK nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to TWIK protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

The invention also provides TWIK chimeric or fusion proteins. As used herein, a TWIK "chimeric protein" or "fusion protein" comprises a TWIK polypeptide operatively linked to a non-TWIK polypeptide. An "TWIK polypeptide" refers to a polypeptide having an amino acid sequence corresponding to TWIK, whereas a "non-TWIK polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the TWIK protein, *e.g.*, a protein which is different from the TWIK protein and which is derived from the same or a different organism. Within a TWIK fusion protein the TWIK polypeptide can correspond to all or a portion of a TWIK protein. In a preferred embodiment, a TWIK fusion protein comprises at least one biologically active portion of a TWIK protein. In another preferred embodiment, a TWIK fusion protein comprises at least two biologically active portions of a TWIK protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the TWIK polypeptide and the non-TWIK polypeptide are fused in-frame to each other. The non-TWIK polypeptide can be fused to the N-terminus or C-terminus of the TWIK polypeptide.

For example, in one embodiment, the fusion protein is a GST-TWIK fusion protein in which the TWIK sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant TWIK.

In another embodiment, the fusion protein is a TWIK protein containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of TWIK can be increased through use of a heterologous signal sequence.

The TWIK fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. The TWIK fusion proteins can be used to affect the bioavailability of a TWIK substrate. Use of TWIK fusion proteins may be useful therapeutically for the treatment of CNS disorders, *e.g.*, neurodegenerative disorders such as Alzheimer's disease, dementias related to Alzheimer's disease (such as Pick's disease), Parkinson's and other Lewy diffuse body diseases, multiple sclerosis, amyotrophic lateral sclerosis, progressive supranuclear palsy, epilepsy and Jakob-Creutzfieldt disease; psychiatric disorders, *e.g.*, depression, schizophrenic disorders, korsakoff's psychosis, mania, anxiety disorders, or phobic disorders; learning or memory disorders, *e.g.*, amnesia or age-related memory loss; neurological disorders; *e.g.*, migraine; and obesity.

Moreover, the TWIK-fusion proteins of the invention can be used as immunogens to produce anti-TWIK antibodies in a subject, to purify TWIK ligands and in screening assays to identify molecules which inhibit the interaction of TWIK with a TWIK substrate.

Preferably, a TWIK chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A TWIK-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the TWIK protein.

The present invention also pertains to variants of the TWIK proteins which function as either TWIK agonists (mimetics) or as TWIK antagonists. Variants of the TWIK proteins can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of a TWIK

protein. An agonist of the TWIK proteins can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of a TWIK protein. An antagonist of a TWIK protein can inhibit one or more of the activities of the naturally occurring form of the TWIK protein by, for example, competitively modulating a potassium channel mediated activity of a TWIK protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the TWIK protein.

In one embodiment, variants of a TWIK protein which function as either TWIK agonists (mimetics) or as TWIK antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of a TWIK protein for TWIK protein agonist or antagonist activity. In one embodiment, a variegated library of TWIK variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of TWIK variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential TWIK sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of TWIK sequences therein. There are a variety of methods which can be used to produce libraries of potential TWIK variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential TWIK sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of a TWIK protein coding sequence can be used to generate a variegated population of TWIK fragments for screening and subsequent selection of variants of a TWIK protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a TWIK coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the TWIK protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of TWIK proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify TWIK variants (Arkin and Yourvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delgrave *et al.* (1993) *Protein Engineering* 6(3):327-331).

In one embodiment, cell based assays can be exploited to analyze a variegated TWIK library. For example, a library of expression vectors can be transfected into a cell line which ordinarily synthesizes TWIK. The transfected cells are then cultured such that TWIK and a particular mutant TWIK are expressed and the effect of expression of the mutant on TWIK activity in the cells can be detected, *e.g.*, by any of a number of enzymatic assays or by detecting the release of a neurotransmitter. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of TWIK activity, and the individual clones further characterized.

An isolated TWIK protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind TWIK using standard techniques for polyclonal and monoclonal antibody preparation. A full-length TWIK protein can be used or, alternatively, the invention provides antigenic peptide fragments of TWIK for use as immunogens. The antigenic peptide of TWIK comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:11 and encompasses an epitope of TWIK such that an antibody raised against the peptide forms a specific immune complex with TWIK. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

Preferred epitopes encompassed by the antigenic peptide are regions of TWIK that are located on the surface of the protein, *e.g.*, hydrophilic regions, as well as regions with high antigenicity (see, for example, Figures 2, 4, 6, and 18).

A TWIK immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed

TWIK protein or a chemically synthesized TWIK polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic TWIK preparation induces a polyclonal anti-TWIK antibody response.

Accordingly, another aspect of the invention pertains to anti-TWIK antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as TWIK. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind TWIK. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of TWIK. A monoclonal antibody composition thus typically displays a single binding affinity for a particular TWIK protein with which it immunoreacts.

Polyclonal anti-TWIK antibodies can be prepared as described above by immunizing a suitable subject with a TWIK immunogen. The anti-TWIK antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized TWIK. If desired, the antibody molecules directed against TWIK can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti-TWIK antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem* .255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci. USA* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a TWIK immunogen as described

above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds TWIK.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-TWIK monoclonal antibody (see, *e.g.*, G. Galfre *et al.* (1977) *Nature* 266:55052; Gefter *et al.* *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind TWIK, *e.g.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-TWIK antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with TWIK to thereby isolate immunoglobulin library members that bind TWIK. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT International Publication No. WO 92/18619; Dower *et al.* PCT International Publication No. WO 91/17271; Winter *et al.* PCT International Publication WO 92/20791; Markland *et al.* PCT International Publication No. WO 92/15679; Breitling *et al.* PCT International Publication WO 93/01288; McCafferty *et al.* PCT International Publication No. WO 92/01047; Garrard *et al.* PCT International Publication No. WO 92/09690; Ladner *et al.* PCT International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology*

9:1370-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al. Nature* (1990) 348:552-554.

Additionally, recombinant anti-TWIK antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Application No. PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT International Publication No. WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

An anti-TWIK antibody (*e.g.*, monoclonal antibody) can be used to isolate TWIK by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-TWIK antibody can facilitate the purification of natural TWIK from cells and of recombinantly produced TWIK expressed in host cells. Moreover, an anti-TWIK antibody can be used to detect TWIK protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the TWIK protein. Anti-TWIK antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine,

dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

III. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding a TWIK protein (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide

sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, TWIK proteins, mutant forms of TWIK proteins, fusion proteins, and the like).

The recombinant expression vectors of the invention can be designed for expression of TWIK proteins in prokaryotic or eukaryotic cells. For example, TWIK proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Purified fusion proteins can be utilized in TWIK activity assays, (*e.g.*, direct assays or competitive assays described in detail below), or to generate antibodies specific for TWIK proteins, for example. In a preferred embodiment, a TWIK fusion protein expressed in a retroviral expression vector of the present invention can be utilized to infect bone marrow cells which are subsequently transplanted into irradiated recipients. The pathology of the subject recipient is then examined after sufficient time has passed (*e.g.*, six (6) weeks).

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990)

60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the TWIK expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.*, (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp, San Diego, CA).

Alternatively, TWIK proteins can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277),

lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to TWIK mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, a TWIK protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and

"transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a TWIK protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a TWIK protein. Accordingly, the invention further provides methods for producing a TWIK protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding a TWIK protein has been introduced) in a suitable medium such that a TWIK protein is produced. In another embodiment, the method further comprises isolating a TWIK protein from the medium or the host cell.

The host cells of the invention can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which TWIK-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous TWIK sequences have been introduced into their genome or homologous recombinant animals in which endogenous TWIK sequences have been altered. Such animals are useful for studying the function and/or activity of a TWIK and for identifying and/or evaluating modulators of TWIK activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby

directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous TWIK gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing a TWIK-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The TWIK cDNA sequence of SEQ ID NO:1 can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homologue of a human TWIK gene, such as a mouse or rat TWIK gene, can be used as a transgene. Alternatively, a TWIK gene homologue, such as another TWIK potassium channel family member, can be isolated based on hybridization to the TWIK cDNA sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12 or the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____ (described further in subsection I above) and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to a TWIK transgene to direct expression of a TWIK protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of a TWIK transgene in its genome and/or expression of TWIK mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding a TWIK protein can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a TWIK gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the TWIK gene. The TWIK gene can be a human gene (*e.g.*, the cDNA of SEQ ID NO:3, 6, 9, or 12), but more preferably, is a non-human homologue of a human TWIK gene (*e.g.*, a cDNA isolated by stringent hybridization with the nucleotide sequence of SEQ ID NO:1, 4, 7, or 10). For example, a mouse TWIK gene can be used to construct a homologous recombination vector suitable for

altering an endogenous TWIK gene in the mouse genome. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous TWIK gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous TWIK gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous TWIK protein). In the homologous recombination vector, the altered portion of the TWIK gene is flanked at its 5' and 3' ends by additional nucleic acid sequence of the TWIK gene to allow for homologous recombination to occur between the exogenous TWIK gene carried by the vector and an endogenous TWIK gene in an embryonic stem cell. The additional flanking TWIK nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced TWIK gene has homologously recombined with the endogenous TWIK gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double"

transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. *et al.* (1997) *Nature* 385:810-813 and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G_0 phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

IV. Pharmaceutical Compositions

The TWIK nucleic acid molecules, fragments of TWIK proteins, and anti-TWIK antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose.

pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a fragment of a TWIK protein or an anti-TWIK antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The

tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular

therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of protein or polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody, protein, or polypeptide in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody, protein, or polypeptide used for treatment may increase or decrease over the course of a particular treatment. Changes in

dosage may result and become apparent from the results of diagnostic assays as described herein.

The present invention encompasses agents which modulate expression or activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention.

Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein.

When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic

injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

V. Uses and Methods of the Invention

The nucleic acid molecules, proteins, protein homologues, and antibodies described herein can be used in one or more of the following methods: a) screening assays; b) predictive medicine (*e.g.*, diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenetics); and c) methods of treatment (*e.g.*, therapeutic and prophylactic). As described herein, a TWIK protein of the invention has one or more of the following activities: (1) it can modulate the release of neurotransmitters, (2) it can modulate membrane excitability, (3) it can influence the resting potential of membranes, (4) it can modulate wave forms and frequencies of action potentials, (5) it can modulate thresholds of excitation, and (6) it can modulate processes which underlie learning and memory, such as integration of sub-threshold synaptic responses and the conductance of back-propagating action potentials, and, thus, can be used to, for example, (1) modulate the release of neurotransmitters, (2) modulate membrane excitability, (3) influence the resting potential of membranes, (4) modulate wave forms and frequencies of action potentials, (5) modulate thresholds of excitation, and (6) modulate processes which underlie learning and memory, such as integration of sub-threshold synaptic responses and the conductance of back-propagating action potentials.

The isolated nucleic acid molecules of the invention can be used, for example, to express TWIK protein (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect TWIK mRNA (*e.g.*, in a biological sample) or a genetic alteration in a TWIK gene, and to modulate TWIK activity, as described further below. The TWIK proteins can be used to treat disorders characterized by insufficient or excessive production of a TWIK substrate or production of TWIK inhibitors. In addition, the TWIK proteins can be used to screen for naturally occurring TWIK substrates, to screen for drugs or compounds which modulate TWIK activity, as well as to treat disorders characterized by insufficient or excessive production of TWIK protein or production of TWIK protein forms which have decreased or aberrant activity compared to TWIK wild type protein (*e.g.*, CNS disorders such as neurodegenerative disorders, *e.g.*, Alzheimer's disease, dementias related to Alzheimer's disease (such as Pick's disease), Parkinson's and other Lewy diffuse body

diseases, multiple sclerosis, amyotrophic lateral sclerosis, progressive supranuclear palsy, epilepsy and Jakob-Creutzfieldt disease; psychiatric disorders, *e.g.*, depression, schizophrenic disorders, korsakoff's psychosis, mania, anxiety disorders, or phobic disorders; learning or memory disorders, *e.g.*, amnesia or age-related memory loss; neurological disorders, *e.g.*, migraine; obesity; and cardiac disorders, *e.g.*, cardiac arrythmia). Moreover, the anti-TWIK antibodies of the invention can be used to detect and isolate TWIK proteins, regulate the bioavailability of TWIK proteins, and modulate TWIK activity.

A. Screening Assays:

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules or other drugs) which bind to TWIK proteins, have a stimulatory or inhibitory effect on, for example, TWIK expression or TWIK activity, or have a stimulatory or inhibitory effect on, for example, the expression or activity of TWIK substrate.

In one embodiment, the invention provides assays for screening candidate or test compounds which are substrates of a TWIK protein or polypeptide or biologically active portion thereof. In another embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a TWIK protein or polypeptide or biologically active portion thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409),

plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In one embodiment, an assay is a cell-based assay in which a cell which expresses a TWIK protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate TWIK activity is determined. Determining the ability of the test compound to modulate TWIK activity can be accomplished by monitoring, for example, the release of a neurotransmitter form a cell which expresses TWIK. The cell, for example, can be of mammalian origin. Determining the ability of the test compound to modulate the ability of TWIK to bind to a substrate can be accomplished, for example, by coupling the TWIK substrate with a radioisotope or enzymatic label such that binding of the TWIK substrate to TWIK can be determined by detecting the labeled TWIK substrate in a complex. For example, compounds (*e.g.*, TWIK substrates) can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a compound (*e.g.*, TWIK substrate) to interact with TWIK without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a compound with TWIK without the labeling of either the compound or the TWIK. McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912. As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a compound and TWIK.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a TWIK target molecule (*e.g.*, a TWIK substrate) with a test compound and determining the ability of the test compound to modulate (*e.g.* stimulate or inhibit) the activity of the TWIK target molecule. Determining the ability of the test compound to modulate the activity of a TWIK target molecule can be accomplished, for example, by determining the ability of the TWIK protein to bind to or interact with the TWIK target molecule.

Determining the ability of the TWIK protein or a biologically active fragment thereof, to bind to or interact with a TWIK target molecule can be accomplished by one of the methods described above for determining direct binding. In a preferred embodiment, determining the ability of the TWIK protein to bind to or interact with a TWIK target

molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target molecule can be determined by detecting induction of a cellular second messenger of the target (*i.e.*, intracellular Ca^{2+}, diacylglycerol, IP_3, and the like), detecting catalytic/enzymatic activity of the target an appropriate substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a target-regulated cellular response.

In yet another embodiment, an assay of the present invention is a cell-free assay in which a TWIK protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the TWIK protein or biologically active portion thereof is determined. Preferred biologically active portions of the TWIK proteins to be used in assays of the present invention include fragments which participate in interactions with non-TWIK molecules, *e.g.*, cyclic nucleotides, or fragments with high surface probability scores (see, for example, Figures 2, 4, 6, and 18). Binding of the test compound to the TWIK protein can be determined either directly or indirectly as described above. In a preferred embodiment, the assay includes contacting the TWIK protein or biologically active portion thereof with a known compound which binds TWIK to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a TWIK protein, wherein determining the ability of the test compound to interact with a TWIK protein comprises determining the ability of the test compound to preferentially bind to TWIK or biologically active portion thereof as compared to the known compound.

In another embodiment, the assay is a cell-free assay in which a TWIK protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the TWIK protein or biologically active portion thereof is determined. Determining the ability of the test compound to modulate the activity of a TWIK protein can be accomplished, for example, by determining the ability of the TWIK protein to bind to a TWIK target molecule by one of the methods described above for determining direct binding. Determining the ability of the TWIK protein to bind to a TWIK target molecule can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA). Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705. As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In an alternative embodiment, determining the ability of the test compound to modulate the activity of a TWIK protein can be accomplished by determining the ability of

the TWIK protein to further modulate the activity of a downstream effector of a TWIK target molecule. For example, the activity of the effector molecule on an appropriate target can be determined or the binding of the effector to an appropriate target can be determined as previously described.

In yet another embodiment, the cell-free assay involves contacting a TWIK protein or biologically active portion thereof with a known compound which binds the TWIK protein to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the TWIK protein, wherein determining the ability of the test compound to interact with the TWIK protein comprises determining the ability of the TWIK protein to preferentially bind to or modulate the activity of a TWIK target molecule.

The cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of isolated proteins (*e.g.*, TWIK proteins or biologically active portions thereof). In the case of cell-free assays in which a membrane-bound form an isolated protein is used (*e.g.*, a potassium channel) it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the isolated protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either TWIK or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to a TWIK protein, or interaction of a TWIK protein with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/ TWIK fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or TWIK protein, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads,

complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of TWIK binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either a TWIK protein or a TWIK target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated TWIK protein or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with TWIK protein or target molecules but which do not interfere with binding of the TWIK protein to its target molecule can be derivatized to the wells of the plate, and unbound target or TWIK protein trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the TWIK protein or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the TWIK protein or target molecule.

In another embodiment, modulators of TWIK expression are identified in a method wherein a cell is contacted with a candidate compound and the expression of TWIK mRNA or protein in the cell is determined. The level of expression of TWIK mRNA or protein in the presence of the candidate compound is compared to the level of expression of TWIK mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of TWIK expression based on this comparison. For example, when expression of TWIK mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of TWIK mRNA or protein expression. Alternatively, when expression of TWIK mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of TWIK mRNA or protein expression. The level of TWIK mRNA or protein expression in the cells can be determined by methods described herein for detecting TWIK mRNA or protein.

In yet another aspect of the invention, the TWIK proteins can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with TWIK ("TWIK-binding proteins" or "TWIK-bp") and are involved in TWIK activity. Such TWIK-binding proteins are also likely to be involved in the propagation of signals by the

TWIK proteins or TWIK targets as, for example, downstream elements of a TWIK-mediated signaling pathway. Alternatively, such TWIK-binding proteins are likely to be TWIK inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for a TWIK protein is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming a TWIK-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the TWIK protein.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a TWIK modulating agent, an antisense TWIK nucleic acid molecule, a TWIK-specific antibody, or a TWIK-binding partner) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

B. Detection Assays

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) map their respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

1. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. This process is called chromosome mapping. Accordingly, portions or fragments of the TWIK nucleotide sequences, described herein, can be used to map the location of the TWIK genes on a chromosome. The mapping of the TWIK sequences to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, TWIK genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the TWIK nucleotide sequences. Computer analysis of the TWIK sequences can be used to predict primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the TWIK sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (*e.g.*, human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio P. *et al.* (1983) *Science* 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the TWIK nucleotide sequences to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a TWIK sequence to its chromosome include *in situ* hybridization (described in Fan, Y. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical such as colcemid that disrupts the mitotic spindle. The

chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York 1988).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between a gene and a disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. *et al.* (1987) *Nature,* 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the TWIK gene, can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

2. Tissue Typing

The TWIK sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags"

which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the TWIK nucleotide sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The TWIK nucleotide sequences of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, or SEQ ID NO:10 can comfortably provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences, such as those in SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, or SEQ ID NO:12 are used, a more appropriate number of primers for positive individual identification would be 500-2,000.

If a panel of reagents from TWIK nucleotide sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

3. Use of Partial TWIK Sequences in Forensic Biology

DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, *e.g.*, hair or skin, or body fluids, *e.g.*, blood,

saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, *e.g.*, PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (*i.e.* another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to non-coding regions of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, or SEQ ID NO:10 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the TWIK nucleotide sequences or portions thereof, *e.g.*, fragments derived from the non-coding regions of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, or SEQ ID NO:10, having a length of at least 20 bases, preferably at least 30 bases.

The TWIK nucleotide sequences described herein can further be used to provide polynucleotide reagents, *e.g.*, labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, *e.g.*, brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such TWIK probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, *e.g.*, TWIK primers or probes can be used to screen tissue culture for contamination (*i.e.* screen for the presence of a mixture of different types of cells in a culture).

C. Predictive Medicine:

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining TWIK protein and/or nucleic acid expression as well as TWIK activity, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant TWIK expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with TWIK protein, nucleic acid expression or activity. For example, mutations in a TWIK gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby phophylactically treat an individual prior to the

onset of a disorder characterized by or associated with TWIK protein, nucleic acid expression or activity.

Another aspect of the invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of TWIK in clinical trials.

These and other agents are described in further detail in the following sections.

1. Diagnostic Assays

An exemplary method for detecting the presence or absence of TWIK protein or nucleic acid in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting TWIK protein or nucleic acid (*e.g.*, mRNA, genomic DNA) that encodes TWIK protein such that the presence of TWIK protein or nucleic acid is detected in the biological sample. A preferred agent for detecting TWIK mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to TWIK mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length TWIK nucleic acid, such as the nucleic acid of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, or the DNA insert of the plasmid deposited with ATCC as Accession Number _____ or _____, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to TWIK mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

A preferred agent for detecting TWIK protein is an antibody capable of binding to TWIK protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or $F(ab')_2$) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect TWIK mRNA, protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of TWIK mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of TWIK protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of TWIK

genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of TWIK protein include introducing into a subject a labeled anti-TWIK antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A preferred biological sample is a serum sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting TWIK protein, mRNA, or genomic DNA, such that the presence of TWIK protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of TWIK protein, mRNA or genomic DNA in the control sample with the presence of TWIK protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of TWIK in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting TWIK protein or mRNA in a biological sample; means for determining the amount of TWIK in the sample; and means for comparing the amount of TWIK in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect TWIK protein or nucleic acid.

2. Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant TWIK expression or activity. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with a misregulation in TWIK protein activity or nucleic acid expression, such as a CNS disorder. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing a disorder associated with a misregulation in TWIK protein activity or nucleic acid expression, such as a CNS disorder. Thus, the present invention provides a method for identifying a disease or disorder associated with aberrant TWIK expression or activity in which a test sample is obtained from a subject and TWIK protein or nucleic acid (*e.g.*, mRNA or genomic DNA) is detected, wherein the presence of TWIK protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant TWIK expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a

subject of interest. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue.

Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant TWIK expression or activity. For example, such methods can be used to determine whether a subject can be effectively treated with an agent for a CNS disorder. Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant TWIK expression or activity in which a test sample is obtained and TWIK protein or nucleic acid expression or activity is detected (*e.g.*, wherein the abundance of TWIK protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant TWIK expression or activity).

The methods of the invention can also be used to detect genetic alterations in a TWIK gene, thereby determining if a subject with the altered gene is at risk for a disorder characterized by misregulation in TWIK protein activity or nucleic acid expression, such as a CNS disorder. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding a TWIK-protein, or the mis-expression of the TWIK gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a TWIK gene; 2) an addition of one or more nucleotides to a TWIK gene; 3) a substitution of one or more nucleotides of a TWIK gene, 4) a chromosomal rearrangement of a TWIK gene; 5) an alteration in the level of a messenger RNA transcript of a TWIK gene, 6) aberrant modification of a TWIK gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a TWIK gene, 8) a non-wild type level of a TWIK-protein, 9) allelic loss of a TWIK gene, and 10) inappropriate post-translational modification of a TWIK-protein. As described herein, there are a large number of assays known in the art which can be used for detecting alterations in a TWIK gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the TWIK-gene (see Abravaya *et al.* (1995) *Nucleic Acids Res* .23:675-682). This method can include the steps of collecting a sample of cells from a subject,

isolating nucleic acid (*e.g.*, genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a TWIK gene under conditions such that hybridization and amplification of the TWIK-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. *et al.*, (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D.Y. *et al.*, (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. *et al.* (1988) *Bio-Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a TWIK gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in TWIK can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M.T. *et al.* (1996) *Human Mutation* 7: 244-255; Kozal, M.J. *et al.* (1996) *Nature Medicine* 2: 753-759). For example, genetic mutations in TWIK can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M.T. *et al. supra.* Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the TWIK gene and detect mutations by comparing the

sequence of the sample TWIK with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) *Proc. Natl. Acad. Sci. USA* 74:560) or Sanger ((1977) *Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the TWIK gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by hybridizing (labeled) RNA or DNA containing the wild-type TWIK sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) *Proc. Natl Acad Sci USA* 85:4397; Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in TWIK cDNAs obtained from samples of cells. For example, the mutY enzyme of E. coli cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a TWIK sequence, *e.g.*, a wild-type TWIK sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in TWIK genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type

nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA*: 86:2766, see also Cotton (1993) *Mutat. Res.* 285:125-144; and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control TWIK nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys Chem* 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl Acad. Sci USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA*

88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, *e.g.,* in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a TWIK gene.

Furthermore, any cell type or tissue in which TWIK is expressed may be utilized in the prognostic assays described herein.

3. Monitoring of Effects During Clinical Trials

Monitoring the influence of agents (*e.g.*, drugs) on the expression or activity of a TWIK protein (*e.g.*, the modulation of membrane excitability or resting potential) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase TWIK gene expression, protein levels, or upregulate TWIK activity, can be monitored in clinical trials of subjects exhibiting decreased TWIK gene expression, protein levels, or downregulated TWIK activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease TWIK gene expression, protein levels, or downregulate TWIK activity, can be monitored in clinical trials of subjects exhibiting increased TWIK gene expression, protein levels, or upregulated TWIK activity. In such clinical trials, the expression or activity of a TWIK gene, and preferably, other genes that have been implicated in, for example, a potassium channel associated disorder can be used as a "read out" or markers of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including TWIK, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) which modulates TWIK activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on potassium channel associated disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of TWIK and other genes implicated in the potassium channel associated disorder, respectively. The levels of gene expression (*e.g.*, a gene expression pattern) can be quantified by northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of TWIK or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) including the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a TWIK protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the TWIK protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the TWIK protein, mRNA, or genomic DNA in the pre-administration sample with the TWIK protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of TWIK to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of TWIK to lower levels than detected, *i.e.* to decrease the effectiveness of the agent. According to such an embodiment, TWIK expression or activity may be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

C. Methods of Treatment:

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant TWIK expression or activity. With regards to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers the study of how a patient's genes determine his or her response to a drug (*e.g.*, a patient's "drug response phenotype", or "drug response genotype".) Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the TWIK molecules of the present invention or TWIK modulators according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant TWIK expression or activity, by administering to the subject a TWIK or an agent which modulates TWIK expression or at least one TWIK activity. Subjects at risk for a disease which is caused or contributed to by aberrant TWIK expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the TWIK aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of TWIK aberrancy, for example, a TWIK, TWIK agonist or TWIK antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating TWIK expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell with a TWIK or agent that modulates one or more of the activities of TWIK protein activity associated with the cell. An agent that modulates TWIK protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring target molecule of a TWIK protein (*e.g.*, a TWIK substrate), a TWIK antibody, a TWIK agonist or antagonist, a peptidomimetic of a TWIK agonist or antagonist, or other small molecule. In one embodiment, the agent stimulates one or more TWIK activities. Examples of such stimulatory agents include active TWIK protein and a nucleic acid molecule encoding TWIK that has been introduced into the cell. In another embodiment, the agent inhibits one or more TWIK activities. Examples of such inhibitory agents include antisense TWIK nucleic acid molecules, anti-TWIK antibodies, and TWIK inhibitors. These modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity of a TWIK protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) TWIK expression or activity. In another embodiment, the method involves administering a TWIK protein or nucleic acid molecule as therapy to compensate for reduced or aberrant TWIK expression or activity.

A preferred embodiment of the present invention involves a method for treatment of a TWIK associated disease or disorder which includes the step of administering a

therapeutically effective amount of a TWIK antibody to a subject. As defined herein, a therapeutically effective amount of antibody (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result from the results of diagnostic assays as described herein.

Stimulation of TWIK activity is desirable in situations in which TWIK is abnormally downregulated and/or in which increased TWIK activity is likely to have a beneficial effect. For example, stimulation of TWIK activity is desirable in situations in which a TWIK is downregulated and/or in which increased TWIK activity is likely to have a beneficial effect. Likewise, inhibition of TWIK activity is desirable in situations in which TWIK is abnormally upregulated and/or in which decreased TWIK activity is likely to have a beneficial effect.

3. Pharmacogenomics

The TWIK molecules of the present invention, as well as agents, or modulators which have a stimulatory or inhibitory effect on TWIK activity (*e.g.*, TWIK gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) potassium channel associated disorders (*e.g.*, CNS disorders such as neurodegenerative disorders, *e.g.*, Alzheimer's disease, dementias related to Alzheimer's disease (such as Pick's disease), Parkinson's and other Lewy diffuse body diseases, multiple sclerosis, amyotrophic lateral sclerosis, progressive supranuclear palsy, epilepsy, and Jakob-Creutzfieldt disease; psychiatric disorders, *e.g.*, depression, schizophrenic disorders, korsakoff's psychosis, mania, anxiety disorders, or phobic disorders; learning or memory disorders, *e.g.*, amnesia or age-related memory loss; neurological disorders; *e.g.*, migraine; and obesity) associated with aberrant TWIK activity. In conjunction with such treatment, pharmacogenomics (*i.e.*, the study of the relationship between an individual's genotype and that individual's response to a foreign

compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer a TWIK molecule or TWIK modulator as well as tailoring the dosage and/or therapeutic regimen of treatment with a TWIK molecule or TWIK modulator.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. *et al.* (1996) *Clin. Exp. Pharmacol. Physiol.* 23(10-11) :983-985 and Linder, M.W. *et al.* (1997) *Clin. Chem.* 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare genetic defects or as naturally-occurring polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association", relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (*e.g.*, a "bi-allelic" gene marker map which consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants.) Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high resolution map can be generated from a combination of some ten-million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process, however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

Alternatively, a method termed the "candidate gene approach", can be utilized to identify genes that predict drug response. According to this method, if a gene that encodes a drugs target is known (*e.g.*, a TWIK protein of the present invention), all common variants

of that gene can be fairly easily identified in the population and it can be determined if having one version of the gene versus another is associated with a particular drug response.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (*e.g.*, N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Alternatively, a method termed the "gene expression profiling", can be utilized to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (*e.g.*, a TWIK molecule or TWIK modulator of the present invention) can give an indication whether gene pathways related to toxicity have been turned on.

Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a TWIK molecule or TWIK modulator, such as a modulator identified by one of the exemplary screening assays described herein.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing are incorporated herein by reference.

EXAMPLES

EXAMPLE 1: IDENTIFICATION AND CHARACTERIZATION OF TWIK-2, TWIK-3, TWIK-4, AND TWIK-5 cDNA

In this example, the identification and characterization of the genes encoding human TWIK-2 (clone Fthka20g4), human TWIK-3 (clone Athua133f10), human TWIK-4 (clone AthTb005e07), and human TWIK-5 are described.

Isolation of the human TWIK2, TWIK-3, TWIK-4 and TWIK-5 cDNA

The invention is based, at least in part, on the discovery of four human genes encoding four novel proteins, referred to herein as TWIK-2, TWIK-3, TWIK-4, and TWIK-5. TWIK-2, TWIK-3, and TWIK-4 were identified as ESTs in a proprietary database based on their homology to the N-terminal domain of TWIK family members. TWIK-5 was initially identified as an EST in a monkey dorsal root ganglion library. This EST was subsequently used to perform a BLAST search in a proprietary database, thereby identifying Homo sapiens chromosome 10 clone CIT987SK-1143A11 (Accession No. AC005880) and Homo sapiens chromosome 10 clone CIT987SK-1054O2 (Accession No. AC005661). A contig of these clones was formed, the exons were identified (based on their homology to the monkey cDNA) and the human TWIK-5 cDNA was constructed.

To clone the TWIK-5 cDNA, PCR primers are designed based on the constructed TWIK-5 cDNA sequence and used to screen a dorsal root ganglion library. The positive clones identified are sequenced, and the sequences are assembled. The TWIK-5 cDNA can then be cloned into a vector and expressed in a cell, and the activity of the TWIK-5 protein can then be determined using any of the assays described herein.

The nucleotide sequence encoding the human TWIK-2 protein is shown in Figure 1 and is set forth as SEQ ID NO:1. The full length protein encoded by this nucleic acid comprises about 499 amino acids and has the amino acid sequence shown in Figure 1 and set forth as SEQ ID NO:2. The coding region (open reading frame) of SEQ ID NO:1 is set forth as SEQ ID NO:3. Clone Fthka20g4, comprising the entire coding region of human TWIK-2 was deposited with the American Type Culture Collection (ATCC®), 10801 University Boulevard, Manassas, VA 20110-2209, on _____, and assigned Accession No. _____.

The nucleotide sequence encoding the human TWIK-3 protein is shown in Figure 3 and is set forth as SEQ ID NO:4. The full length protein encoded by this nucleic acid comprises about 332 amino acids and has the amino acid sequence shown in Figure 3 and set forth as SEQ ID NO:5. The coding region (open reading frame) of SEQ ID NO:4 is set forth as SEQ ID NO:6. Clone Athua133f10, comprising the entire coding region of human

TWIK-3 was deposited with the American Type Culture Collection (ATCC®), 10801 University Boulevard, Manassas, VA 20110-2209, on _____, and assigned Accession No. _____.

The nucleotide sequence encoding the human TWIK-4 protein is shown in Figure 5 and is set forth as SEQ ID NO:7. The full length protein encoded by this nucleic acid comprises about 313 amino acids and has the amino acid sequence shown in Figure 5 and set forth as SEQ ID NO:8. The coding region (open reading frame) of SEQ ID NO:7 is set forth as SEQ ID NO:9. Clone AthTb005e07, comprising the entire coding region of human TWIK-4 was deposited with the American Type Culture Collection (ATCC®), 10801 University Boulevard, Manassas, VA 20110-2209, on _____, and assigned Accession No. _____.

The nucleotide sequence encoding the human TWIK-5 protein is shown in Figure 17 and is set forth as SEQ ID NO:10. The full length protein encoded by this nucleic acid comprises about 401 amino acids and has the amino acid sequence shown in Figure 17 and set forth as SEQ ID NO:11. The coding region (open reading frame) of SEQ ID NO:10 is set forth as SEQ ID NO:12.

Analysis of human TWIK2, TWIK-3, TWIK-4, and TWIK-5

A BLASTX 1.4 search, using a score of 100 and a word length of 3 (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403) of the translated nucleotide sequence of human TWIK-2 revealed that human TWIK-2 is similar to the human TASK-2 protein (Accession Number AF084830) . The human TWIK-2 protein is 97% identical to the human TASK-2 protein (Accession Number AF084830) over translated nucleotides 10 to 957 and 90% identical to the human TASK-2 protein (Accession Number AF084830) over translated nucleotides 1042 to 1506.

A BLASTN 1.4.9 search, using a score of 100 and a word length of 12 (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403) of the nucleotide sequence of human TWIK-2 revealed that TWIK-2 is similar to the nucleic acid encoding the human TASK-2 protein (Accession Number AF084830). The TWIK-2 nucleic acid molecule is 98% identical to the nucleic acid encoding the human TASK-2 protein (Accession Number AF084830) over nucleotides 1 to 1644, 99% identical over nucleotides 1590 to 2518, and 99% identical over nucleotides 2788 to 3426.

A BLASTX 1.4 search, using a score of 100 and a word length of 3 (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403) of the translated nucleotide sequence of human TWIK-3 revealed that human TWIK-3 is similar to the human TASK-2 protein (Accession Number AF084830) . The human TWIK-3 protein is 42% identical to the human TASK-2 protein (Accession Number AF084830) over translated nucleotides 719 to 982 and 37% identical to

the human TASK-2 protein (Accession Number AF084830) over translated nucleotides 353 to 577.

A BLASTN 1.4.9 search, using a score of 100 and a word length of 12 (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403) of the nucleotide sequence of human TWIK-3 revealed that TWIK-3 is similar to the human genomic clone 2385B13 (Accession Number AQ240175). The TWIK-3 nucleic acid molecule is 98% identical to the human genomic clone 2385B13 (Accession Number AQ240175) over nucleotides 1176 to 1544.

A BLASTX 1.4 search, using a score of 100 and a word length of 3 (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403) of the translated nucleotide sequence of human TWIK-4 revealed that human TWIK-4 is similar to the human TWIK-1 protein (Accession Number U33632). The human TWIK-4 protein is 53% identical to the human TWIK-1 protein (Accession Number U33632) over translated nucleotides 406 to 837, and 42% identical to over translated nucleotides 220 to 390.

A BLASTN 1.4.9 search, using a score of 100 and a word length of 12 (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403) of the nucleotide sequence of human TWIK-4 revealed that TWIK-4 is similar to the human potassium channel KCNO1 mRNA (Accession Number U90065). The TWIK-4 nucleic acid molecule is 63% identical to the human potassium channel KCNO1 mRNA (Accession Number U90065) over nucleotides 405 to 905.

The TWIK-2 protein was aligned with the TWIK-3 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4. The results showed a 32.166% identity and 40.764% similarity between the two protein sequences (see Figure 10).

The TWIK-2 protein was also aligned with the TWIK-4 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4. The results showed a 27.333% identity and 37.667% similarity between the two protein sequences (see Figure 11).

The TWIK-3 protein was further aligned with the TWIK-4 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4. The results showed a 31.667% identity and 40.333% similarity between the two protein sequences (see Figure 12).

The TWIK-1 protein was aligned with the TWIK-2 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4. The results showed a 26.433% identity and 36.943% similarity between the two protein sequences (see Figure 13).

The TWIK-1 protein was also aligned with the TWIK-3 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a

length weight of 4. The results showed a 22.961% identity and 31.420% similarity between the two protein sequences (see Figure 14).

The TWIK-1 protein was further aligned with the TWIK-4 protein using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4. The results showed a 46.284% identity and 55.405% similarity between the two protein sequences (see Figure 15).

The TWIK-2 protein was aligned with the hTASK-2 protein (described in Reyer R. *et al.* (1998) *J. Biol. Chem.* 273(47):30863-30869) using the GAP program in the GCG software package (Blosum 62 matrix) and a gap weight of 12 and a length weight of 4. The results showed a 100% identity and 100% similarity between the two protein sequences (see Figure 16).

The TWIK-5 protein was aligned with the hTASK-2 protein (described in Reyer R. *et al.* (1998) *J. Biol. Chem.* 273(47):30863-30869) using the GAP program in the GCG software package (PAM250 matrix) and a gap weight of 25 and a length weight of 1. The results showed a 29.183% identity and 49.805% similarity between the two protein sequences (see Figure 19).

The TWIK-5 protein was also aligned with the mouse TREK protein using the GAP program in the GCG software package (PAM250 matrix) and a gap weight of 25 and a length weight of 1. The results showed a 27.273% identity and 44.697% similarity between the two protein sequences (see Figure 20).

A multiple sequence alignment of the amino acid sequence of human TWIK-4, human TWIK-1, murine TRAAK, murine TREK-1, human TWIK-3, human TWIK-2, and human TASK is presented in Figure 7; a multiple sequence alignment of the amino acid sequence of human TWIK-1, human TWIK-4, murine TRAAK, murine TREK-1, and human TASK is presented in Figure 8; and a multiple sequence alignment of the amino acid sequence of human TWIK-3, human TASK2, human TWIK-1, and human TASK is presented in Figure 9.

Hydropathy plots have identified 4 transmembrane domains and two P-loops in each of TWIK-2, TWIK-3, TWIK-4, and TWIK-5 (see figures 2, 4, 6, and 18).

Tissue Distribution of human TWIK2, TWIK-3, TWIK-4, and TWIK-5 mRNA

This Example describes the tissue distribution of human TWIK2, TWIK-3, TWIK-4, and TWIK-5 mRNA, as may be determined by Northern blot hybridization, in situ hybridization, and PCR.

Northern blot hybridizations with the various RNA samples are performed under standard conditions and washed under stringent conditions, *i.e.*, 0.2xSSC at 65°C. The DNA probe is radioactively labeled with ^{32}P-dCTP using the Prime-It kit (Stratagene, La

Jolla, CA) according to the instructions of the supplier. Filters containing human mRNA (MultiTissue Northern I and MultiTissue Northern II from Clontech, Palo Alto, CA) were probed in ExpressHyb hybridization solution (Clontech) and washed at high stringency according to manufacturer's recommendations. Using the above-described methods, it was determined that TWIK-5 is expressed in dorsal root ganglion (DRG) neurons.

TWIK-5 expression in normal human and monkey tissues was assessed by PCR using the Taqman ® system (PE Applied Biosystems) according to the manufacturer's instructions. These studies demonstrated that TWIK-5 is exclusively expressed in monkey DRG neurons.

For *in situ* analysis, various tissues obtained from brains, *e.g.* rat or monkey brains, were first frozen on dry ice. Ten-micrometer-thick coronal sections of the tissues were postfixed with 4% formaldehyde in DEPC treated 1X phosphate- buffered saline at room temperature for 10 minutes before being rinsed twice in DEPC 1X phosphate-buffered saline and once in 0.1 M triethanolamine-HCl (pH 8.0). Following incubation in 0.25% acetic anhydride-0.1 M triethanolamine-HCl for 10 minutes, sections were rinsed in DEPC 2X SSC (1X SSC is 0.15M NaCl plus 0.015M sodium citrate). Tissue was then dehydrated through a series of ethanol washes, incubated in 100% chloroform for 5 minutes, and then rinsed in 100% ethanol for 1 minute and 95% ethanol for 1 minute and allowed to air dry.

Hybridizations were performed with ^{35}S-radiolabeled (5 X 10^7 cpm/ml) cRNA probes. Probes were incubated in the presence of a solution containing 600 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 0.01% sheared salmon sperm DNA, 0.01% yeast tRNA, 0.05% yeast total RNA type X1, 1 X Denhardt's solution, 50% formamide, 10% dextran sulfate, 100 mM dithiothreitol, 0.1% sodium dodecyl sulfate (SDS), and 0.1% sodium thiosulfate for 18 hours at 55°C.

After hybridization, slides were washed with 2X SSC. Sections were then sequentially incubated at 37°C in TNE (a solution containing 10 mM Tris-HCl (pH 7.6), 500 mM NaCl, and 1 mM EDTA), for 10 minutes, in TNE with 10μg of RNase A per ml for 30 minutes, and finally in TNE for 10 minutes. Slides were then rinsed with 2X SSC at room temperature, washed with 2X SSC at 50°C for 1 hour, washed with 0.2X SSC at 55°C for 1 hour, and 0.2X SSC at 60°C for 1 hour. Sections were then dehydrated rapidly through serial ethanol-0.3 M sodium acetate concentrations before being air dried and exposed to Kodak Biomax MR scientific imaging film for 24 hours and subsequently dipped in NB-2 photoemulsion and exposed at 4°C for 7 days before being developed and counter stained. These experiments resulted in the identification of very high levels of TWIK-5 expression in a subpopulation of monkey DRG (small and intermediate size) neurons, and minimal to no expression of TWIK-5 in peripheral tissues. Bright field observation demonstrates that TWIK-5 is expressed in the small diameter neurons that correspond to a subpopulation of nociceptive neurons, and suggests a potential involvement of the gene in the regulation or

maintenance of the electrophysiological characteristics of a subpopulation of nociceptive neurons, and thereby in the modulation of pain responses.

EXAMPLE 2: EXPRESSION OF RECOMBINANT TWIK-2, TWIK-3, TWIK-4, AND TWIK-5 PROTEIN IN BACTERIAL CELLS

In this example, TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 is expressed as a recombinant glutathione-S-transferase (GST) fusion polypeptide in *E. coli* and the fusion polypeptide is isolated and characterized. Specifically, TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 is fused to GST and this fusion polypeptide is expressed in *E. coli*, *e.g.*, strain PEB199. Expression of the GST-TWIK-2, GST-TWIK-3, GST-TWIK-4, and/or GST-TWIK-5 fusion protein in PEB199 is induced with IPTG. The recombinant fusion polypeptide is purified from crude bacterial lysates of the induced PEB199 strain by affinity chromatography on glutathione beads. Using polyacrylamide gel electrophoretic analysis of the polypeptide purified from the bacterial lysates, the molecular weight of the resultant fusion polypeptide is determined.

EXAMPLE 3: EXPRESSION OF RECOMBINANT TWIK-2, TWIK-3, TWIK-4, AND TWIK-5 PROTEIN IN COS CELLS

To express the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 gene in COS cells, the pcDNA/Amp vector by Invitrogen Corporation (San Diego, CA) is used. This vector contains an SV40 origin of replication, an ampicillin resistance gene, an *E. coli* replication origin, a CMV promoter followed by a polylinker region, and an SV40 intron and polyadenylation site. A DNA fragment encoding the entire TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 protein and an HA tag (Wilson *et al.* (1984) *Cell* 37:767) or a FLAG tag fused in-frame to its 3' end of the fragment is cloned into the polylinker region of the vector, thereby placing the expression of the recombinant protein under the control of the CMV promoter.

To construct the plasmid, the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 DNA sequence is amplified by PCR using two primers. The 5' primer contains the restriction site of interest followed by approximately twenty nucleotides of the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 coding sequence starting from the initiation codon; the 3' end sequence contains complementary sequences to the other restriction site of interest, a translation stop codon, the HA tag or FLAG tag and the last 20 nucleotides of the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 coding sequence. The PCR amplified fragment and the pCDNA/Amp vector are digested with the appropriate restriction enzymes and the vector is dephosphorylated using the CIAP enzyme (New England Biolabs, Beverly, MA).

Preferably the two restriction sites chosen are different so that the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 gene is inserted in the correct orientation. The ligation mixture is transformed into *E. coli* cells (strains HB101, DH5a, SURE, available from Stratagene Cloning Systems, La Jolla, CA, can be used), the transformed culture is plated on ampicillin media plates, and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment.

COS cells are subsequently transfected with the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5-pcDNA/Amp plasmid DNA using the calcium phosphate or calcium chloride co-precipitation methods, DEAE-dextran-mediated transfection, lipofection, or electroporation. Other suitable methods for transfecting host cells can be found in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The expression of the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 polypeptide is detected by radiolabelling (^{35}S-methionine or ^{35}S-cysteine available from NEN, Boston, MA, can be used) and immunoprecipitation (Harlow, E. and Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988) using an HA specific monoclonal antibody. Briefly, the cells are labelled for 8 hours with ^{35}S-methionine (or ^{35}S-cysteine). The culture media are then collected and the cells are lysed using detergents (RIPA buffer, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM Tris, pH 7.5). Both the cell lysate and the culture media are precipitated with an HA specific monoclonal antibody. Precipitated polypeptides are then analyzed by SDS-PAGE.

Alternatively, DNA containing the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 coding sequence is cloned directly into the polylinker of the pCDNA/Amp vector using the appropriate restriction sites. The resulting plasmid is transfected into COS cells in the manner described above, and the expression of the TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 polypeptide is detected by radiolabelling and immunoprecipitation using a TWIK-2, TWIK-3, TWIK-4, and/or TWIK-5 specific monoclonal antibody.

EXAMPLE 4: ELECTROPHYSIOLOGICAL CHARACTERIZATION OF TWIK-5

To electrophysiologically characterize the TWIK-5 molecule, the full length human TWIK-5 gene in the pMet7 expression vector was used to transiently transfect CHO cells using lipofectamine. **[TO INVENTORS: PLEASE CONFIRM TRANSFECTION METHODOLOGIES]** Electrophysiological measurements in the transfected CHO cells were taken using a single electrode patch-clamp, 48 hours after transfection. Cell membrane potentials were held at –80mV and then depolarized from –60 mV to +50 mV with 10 mV increments for 50 msec, followed by 20 msec hyperpolarization to –120 msec (see Figure

21A, lower panel). TWIK-5 displayed outward currents from –60 to +50 mV (Figure 21A, upper panel). The amplitude of current at +50 mV was 4 mA. The current-membrane potential curve was linear from +10mV to +50 mV (Figure 21B).

The pharmacological properties of the TWIK-5 channel were studied by incubating a series of known potassium channel inhibitory molecules with the transiently transfected CHO cells, and examining the impact of the molecules upon the conductance of TWIK-5 channels through whole-cell patch-clamp recordings. As shown in Figure 22, the addition of 10 mM Ba^{++} was completely inhibitory to the function of the TWIK-5 channels (Figure 22B), and the presence of 10 mM triethylammonium (Figure 22C) was also significantly inhibitory to TWIK-5 channel conductance, though less so that Ba^{++}. The addition of 10 mM 4-aminopyridine, a compound known to be inhibitory to several voltage-gated potassium channels, had no effect on the conductance of the TWIK-5 channel (Figure 22D).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.